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Diet-shift driven $\delta^{13}C$ and $\delta^{15}N$ changes in liver and muscle tissues of juvenile clownfish *Amphiprion frenatus*: A laboratory experiment

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ABSTRACT

A laboratory-based diet-shift study using juvenile clownfish (*Amphiprion frenatus*) was performed to determine 1) isotopic turnover rates and 2) trophic fractionation of $\delta^{13}C$ and $\delta^{15}N$ in liver and muscle tissues. A brood of *A. frenatus* was first fed shrimps (*Acetes erythraeus*) with $\delta^{13}C = -19.2‰$ and $\delta^{15}N = 8.3‰$ for 29 days to provide an isotopic baseline. Subsequently, they were fed muscle filets from a predatory reef fish (*Sparus latus*) with isotopic signature of $\delta^{13}C = -15.9‰$ and $\delta^{15}N = 11.3‰$. The results showed significant differences in $\delta^{13}C$ and $\delta^{15}N$ between liver and muscle throughout the most of the experiment, indicating different incorporation of isotopes by these two tissues. $\delta^{13}C$ in muscle tissue was less depleted than liver tissue before the diet shift and more depleted after. The change in isotopic composition was significantly faster for liver than muscle tissue after the diet-shift. A hyperbolic saturation model provided a good fit for predicting the time scale of isotopic turnover. The model showed that liver tissue approaches isotopic saturation 3–4 times faster than muscle tissue. Isotopic fractionations extrapolated from the model output at saturation were remarkably similar for liver and muscle tissues ($\Delta^{13}C$ of −0.5 and −0.3‰, and $\Delta^{15}N$ of 2.6 and 2.8‰, respectively), which indicates that fractionation is independent of tissue or organ in *A. frenatus*. Liver tissue therefore appears more useful than muscle tissue as a short-term dietary indicator for *A. frenatus* and possibly also for other fish species.

1. Introduction

Ecologists have long been interested in the trophic structure of marine animal communities and associated nutrient pathways (Rau et al., 1983). For that reason, much research has in recent years focused on foodwebs, which are assumed good indicators of ecosystem functioning (Pasquaud et al. 2007). Carbon and nitrogen stable isotopes are widely used in studies of foodweb structure (Post, 2002; Fry, 2006; Boecklen et al., 2011). They are excellent identifiers of trophic relationships and energy flow patterns, and $\delta^{13}C$ has traditionally been used to identify the food source, while $\delta^{15}N$ reflects the trophic level of a consumer (Sweeting et al., 2007a, 2007b). However, reliable interpretation of stable isotope data requires knowledge of the isotopic fractionation between diet and consumer, which may vary among animal species and their diets (McCutchan et al., 2003). When bulk tissue $\delta^{13}C$ composition is used for diet analysis, the fractionation is typically assumed close to zero (Trueman et al., 2005; McCutchan et al., 2003). Bulk isotopic fractionation of $\delta^{15}N$, on the other hand, is generally considered to be 2–4‰ (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002; McCutchan et al., 2003). The positive change in consumer $\delta^{15}N$ reflects preferential retention of heavier isotopes and excretion of lighter isotopes via production of waste products (e.g. urea, ammonia) (Gannes et al., 1997; Trueman et al., 2005). However, bulk consumer isotope signals are the average of all isotopic fractionation reactions occurring within the body, which may differ among tissues and be influenced by nutritional status, feeding rate, growth and metabolism (DeNiro and Epstein, 1981; Sweeting et al., 2007a, 2007b; Buchheister and Latour, 2010). Although considerable research has been conducted in recent years, the pattern of isotope enrichment between various diets and consumer organs is not thoroughly understood for most taxa (Sweeting et al., 2007a).

Fish are important consumers in aquatic foodwebs and the use of stable isotopes to study interactions among fish is increasing (Pinnegar and Polunin, 1999; Galvan et al., 2012). For most examined fish species, bulk $\delta^{13}C$ and $\delta^{15}N$ fractionations are on average about 1.5 and 2.9‰, respectively, per trophic level, but with considerable inter-species variation among values, i.e. −1.7 to +3.7‰ for $\delta^{13}C$ and −0.3 to +5.6 for $\delta^{15}N$ (Sweeting et al., 2007a, 2007b). Vander Zanden and Rasmussen (2001) similarly reported a wide inter-species range of $\delta^{15}N$ fractionation for aquatic animals in general from −0.7 to +9.2‰. Other factors than species are subject to uncertainty when determining the $\delta^{13}C$ and $\delta^{15}N$ for fish, including size, age, diet composition, and nutritional status as well as the type of tissue sampled from the fish (Gannes et al., 1997; Pasquaud et al., 2007). Temperature and other environmental conditions

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may also influence carbon and nitrogen isotope composition of fish (Barnes et al., 2007; Michener and Lajtha, 2007).

Although the number of studies examining stable isotope turnover rates in fish tissues has increased in recent years (e.g., MacNeil et al., 2006; Buchheister and Latour, 2010) most tissues remain understudied relative to muscle. Fish tissues that are metabolically active, such as liver, tend to respond faster to dietary changes (Buchheister and Latour, 2010) than other less active tissues, such as muscle (MacAvoy et al., 2001). High turnover rates allow shorter time-scales for assessing isotope saturation kinetics and thus to track nutritional changes at a higher resolution. However, the few studies that have compared isotope dynamics of various tissues in fish have found variable turnover rates and trophic enrichments among fish species and tissues, highlighting the need for further research (Trueman et al., 2005; Logan et al., 2008; Buchheister and Latour, 2010).

The aim of this study was to determine $\delta^{13}C$ and $\delta^{15}N$ in muscle and liver tissues of the clownfish, *Amphiprion frenatus*; and to estimate isotopic carbon and nitrogen fractionations of this fish following a change in diet. The study was conducted as a laboratory feeding experiment under controlled environmental conditions, during which *A. frenatus* was fed food items of known amounts and isotopic composition. The hypotheses tested were 1) the isotopic turnover time in liver tissue is shorter than in muscle tissue; and 2) liver and muscle tissues fractionate C and N isotopes similarly. If these hypotheses hold, liver could potentially be faster than muscle tissue for tracing the diet of *A. frenatus* and probably other fish species.

2. Materials and methods

2.1. Fish husbandry

A. frenatus is commonly known as the tomato clownfish and was first described by Brevoort in 1856 (Papavlasopoulou et al., 2014). The preferred habitat for this species is embayments associated with coral reefs. It is non-migratory and has a depth range of 1–12 m in tropical waters from 25°N–35°S. *A. frenatus* was chosen because it can be easily reared in captivity, and therefore is likely to provide reliable isotope results. Furthermore, its high trophic level of 2.7 (Froese and Pauly, 2000) allows for selection of food items with a wide range of stable isotope signals.

The Institute of Oceanography, Vietnam Academy of Science and Technology, Nha Trang, Vietnam routinely cultivates *A. frenatus* in their laboratories. The individuals used in this study were from the same brood to ensure similar age and size. A batch of 60 juvenile individuals with a length of about 3 cm, were held in a 160-liter experimental tank facility (80 × 50 × 40 cm) at the Institute of Oceanography from 4 March to 2 May 2012 (60 days). The tank was supplied with seawater, which was filtered to ensure no food was available, and exposed to natural daylight. All water in the experiment tank was changed about 3–4 times per week. Since the experimental conditions were identical to the initial rearing condition, no acclimation was needed. Temperature was constant throughout the experimental period, but varied every day from 25 °C in the morning to 30 °C in the afternoon. Salinity was 29–33 throughout the study; highest just before and lowest just after water change. No hiding items were available for the fish.

2.2. Experimental procedures and sampling

The experiment was divided into two phases, each lasting about one month. During the baseline phase the fish were fed an old supply of *Acetes erythraeus*, a common and quite abundant shrimp (denoted *Acetes*). The batch of *A. frenatus* has been fed *Acetes* of unknown origin and isotopic composition prior to the experiment. However, the fish did not eat well and appeared distressed during the first couple of weeks of the experiment, and a fresh supply of *Acetes* was used from day 20. The two supplies are referred as *Acetes*-old and *Acetes*-new, respectively (Table 1). *Acetes* were kept frozen throughout the experiment, except when used as food. In the 2nd test phase after day 29, the diet was changed to muscle filets from a single *Sparus latus* (a top predator reef fish) to ensure a consistent change in diet $\delta^{13}C$ and $\delta^{15}N$. The fish filets were cut into few cm long pieces prior to the experiment and stored frozen for the duration of the experiment. In both phases, the fish were fed to satiation twice daily at 8:30 and 14:00. Subsamples of *Acetes*-old, *Acetes*-new and *S. latus* were collected randomly for isotope analysis.

Randomly chosen individuals of *A. frenatus* were caught and killed by freezing on each sampling date. Initial samples were taken on day 1 (n = 5) and subsequently about once every week (n = 3) during the first phase (5 sampling dates) and every third day (n = 3) during the second phase (10 sampling dates). Length of each fish was measured from the snout tip to the end of the tail fin before liver and muscle tissues were dissected using scissors and tweezers. Muscle removal was done with care to avoid scales and bones. The samples were dried in an oven at 60 °C for 96 h and stored frozen (−20 °C) in Eppendorf tubes until transport and further processing at the University of Southern Denmark. Studies have shown that freezing and storage have no effect on $\delta^{13}C$ or $\delta^{15}N$ signals (Sweeting et al., 2006).

2.3. Isotope analysis

The dried samples were homogenized by grinding using pestle and mortar and a subsample of about 0.5–1 mg was taken for $\delta^{13}C$ and $\delta^{15}N$ determination. A continuous flow Thermo Scientific Isotope Ratio Mass Spectrometer was used for isotope analysis with continuous calibrations during analyses. Isotope values are expressed in δ (delta) notations as parts per thousand according to the following equation:

$$\delta X(‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 10^3$$

where X is ^{13}C or ^{15}N, and R the isotopic $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the sample and standard, respectively. All results are reported relative to PeeDee Belemnite limestone carbon and atmospheric nitrogen standards (Aita et al., 2011; Benstead et al., 2006). The precision of $\delta^{13}C$ and $\delta^{15}N$ analyses was ±0.18 and ±0.10‰, respectively.

2.4. Statistical analyses

To test for differences between liver and muscle, a paired-sample t-test was used for $\delta^{13}C$, $\delta^{15}N$ and C/N. The data for $\delta^{15}N$ did not pass the test for equal variance and C/N did not pass the test for normality, so a Mann–Whitney Rank Sum test was conducted for both. All tests were conducted with a confidence interval of p = 0.05. Statistics were performed using SigmaPlot for Windows Version 11.0.

Table 1
$\delta^{13}C$ and $\delta^{15}N$ signals and C/N ratio for *Acetes erythraeus* (old and new), *Sparus latus* muscle tissue as well as muscle and liver tissues of *Amphiprion frenatus*. Values are given as mean ± SD, n = 3 for food items and n = 5 for *A. frenatus*.

	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C/N
Acetes old	−19.7 ± 0.2	8.5 ± 0.7	3.7 ± 0.1
Acetes new	−18.8 ± 0.5	8.1 ± 0.2	3.7 ± 0.1
Sparus latus	−15.9 ± 0.3	11.3 ± 0.2	3.3 ± 0.2
Amphiprion frenatus			
Muscle	−19.5 ± 0.3	14.1 ± 0.4	3.8 ± 0.2
Liver	−20.4 ± 0.3	12.1 ± 0.4	6.2 ± 0.7